Triangle Research Labs, LLC | www.TRLcells.com
U.S. Scientific Support: 800 521 0390 | scientific.support@lonza.com
EU/ROW Scientific Support: +32 87 321 611 | scientific.support.eu@lonza.com

Using Cryopreserved Hepatocytes Protocol

This protocol is suitable for the thawing of suspension and plateable cryopreserved hepatocytes. Please read through this entire protocol before attempting this procedure. The health of the hepatocytes is dependent upon following the protocol carefully.

Procedure for Thawing

1. Warm the appropriate media for your application (refer to Table 1, below) in a 37°C waterbath. *NOTE: Hepatocyte Maintenance Medium (MM250) and Hepatocyte Plating Medium (MP100) must be completed with included supplement prior to use.*

Table 1. Media Requirements by Species and Application Type

Species	Application Type	MCHT50	MCHT50P	MCAT50	MCRT50	MP100	MM250
Human	Single Donor Suspension	√					√
	Pooled Suspension		√				√
	Plated	√				√	√
Dog, Monkey	Suspension			√			√
	Plated			√		√	√
Rat, Mouse	Suspension				√		√
	Plated				√	√	√

2. Once the thawing medium is warmed, disinfect it (70% ethanol wipe or spray) and transfer it to the biological safety cabinet (BSC) (or alternative designated area).
3. Quickly remove the cryopreserved hepatocytes from their storage location (shipping dewar or storage dewar). Vertically submerge as much of the vial as possible, up to the cap, in the waterbath. It is important to make sure the cap of the vial stays above the waterline.
4. Thaw the vial for approximately 90 – 120 seconds. The vial will thaw from the outside to the inside; you can see a spindle form and shrink as the vial thaws.
5. Once thawed, disinfect the vial and transfer it to the BSC. Pour or pipette (with a wide-bore tip) hepatocytes into a 50 mL conical tube of thawing medium. You may pipette approximately 1 mL thawing medium back into the original vial and pour or pipette the remaining cells back into the 50 mL tube of thawing medium to ensure that all hepatocytes are transferred.
6. Suspend the cells by carefully rocking the 50 mL tube in your hands for a few seconds.
7. Centrifuge at room temperature, following the guidelines in Table 2, below.

Table 2. Spin Speed and Duration by Species

Species	Spin Speed (g)	Duration (min)
Human	100	8
Pooled Human	200	10
Mouse	100	4
Rat	100	10
Dog and non-human primate	65	4

8. Remove tube from centrifuge, disinfect and transfer to the BSC, and pour supernatant into a waste bottle, inverting completely, without shaking.
9. For every 1x10^6 total cells expected (refer to the data sheet), add ~1 mL of maintenance medium (for suspension use) or plating medium (for plated applications) to the cell pellet.

Procedure for Suspension Use

10. Determine the viability and yield of your hepatocytes, via Trypan Blue exclusion method (see our Trypan Blue Cell Counting Protocol for assistance).
11. Add additional maintenance medium to bring cells to desired concentration of experimental design (most commonly 1x10^6 cells/mL).
12. It is recommended that you allow the hepatocytes to acclimate for 10 minutes by placing them on an orbital shaker at 120 rpm inside the incubator. Your hepatocytes are now ready to use.

Procedure for Plated Use

Note: Use rat tail type-I collagen-coated plates.

10. Use the formulas below to determine the volume of plating medium to add to your current cell stock to achieve the desired cell density (refer to Table 3, below).

Live cell yield	/	Desired cell density*	=	Total volume needed (mL)
_____ *x 10^6 cells*	/	_____ *x 10^6 cells/mL*	=	_____ *mL*

Total volume needed	-	Current volume	=	Volume to add to cell stock
_____ *mL*	-	_____ *mL*	=	_____ *mL*

Table 3. Desired Cell Density by Species and Plate Format

Species	6-well	12-well	24-well	48-well	96-well
	Cell Density (10^{6}cells/mL)				
Human, Rat, Dog	0.9 – 1.1	0.8 – 1.0	0.7 – 0.9	0.6 – 0.8	0.9 – 1.1
Monkey	1.1 – 1.3	1.0 – 1.2	0.9 – 1.1	0.8 – 1.0	1.1 – 1.3
Mouse	0.5 – 0.7	0.4 – 0.6	0.3 – 0.5	0.2 – 0.4	0.5 – 0.7

11. Using a pipette (preferably with wide-bore tip), transfer hepatocytes to a multi-well plate. Use Table 4 below for correct volume of cell stock to add to each well.
NOTE: For 96-well plates, add 50µL of blank plating media to each well followed by 50µL of cell stock to uniformly disperse hepatocytes.

Table 4. Cell Volume Per Well

Plate Format	6-well	12-well	24-well	48-well	96-well
Cell Volume Per Well (mL/well) – All Species	2.0mL/well	1.0mL/well	0.50mL/well	0.20mL/well	0.050mL blank media/well + 0.050mL/well

Table 5. Approximate Number of Cells per Well

Species	6-well	12-well	24-well	48-well	96-well
	Approximate Number of Cells Per Well				
Human, Dog	2.0×10^6	0.9×10^6	0.40×10^6	0.175×10^6	0.050×10^6
Monkey	2.4×10^6	1.1×10^6	0.50×10^6	0.225×10^6	0.060×10^6
Mouse	1.2×10^6	0.5×10^6	0.20×10^6	0.075×10^6	0.030×10^6
Rat	1.8×10^6	0.8×10^6	0.35×10^6	0.150×10^6	0.045×10^6

NOTE: We recommend you visually check seeding density to ensure a confluent monolayer

12. Place plate in a 37°C/5% CO_2 incubator. For all plate formats except 96-well, disperse the cells by moving the plate, with your hand on top of it, parallel to the incubator shelf in a north-south, east-west motion. Note: For 96-well plates, place directly in the incubator without shaking.
13. Repeat the shaking motion at 15, 30, and 45 minutes post-seeding.
14. At 60 minutes, remove plates from the incubator, carefully aspirate the media, and replace with fresh plating medium (refer to volumes in Table 6 below).
15. Incubate the cells for a total of 4 – 6 hours post-seeding.
16. If using an overlay, proceed to the next section. If not, replace the medium with warm maintenance medium or application specific medium according to your experimental guidelines.
17. Replace maintenance medium daily following Table 6 below.

Table 6. Maintenance Medium Volume Per Well

Plate Format	6-well	12-well	24-well	48-well	96-well
Media Volume Per Well (mL/well) – All Species	2.0mL/well	1.0mL/well	0.50mL/well	0.20mL/well	0.10mL/well

Procedure for Overlay

Overlay matrix and the maintenance medium used for its dilution should be kept at or below 4°C. Keep everything on ice when preparing and while using the overlay.

18. Calculate the volume of maintenance medium needed to feed your plate(s). This is generally 12 mL per plate. Add a few milliliters extra for an excess of overlay solution.
19. Find the protein concentration of the overlay matrix on its specification sheet. Use the formula below to determine how much overlay matrix to add to maintenance medium. We recommend a final overlay matrix concentration between 0.25 mg/mL and 0.35 mg/mL.

(Volume of medium needed X 0.3 mg/mL) / Overlay matrix concentration = Volume of overlay matrix to add

(_____ mL X 0.3 mg/mL)/ _____ mg/mL = _____ mL

20. Add the calculated amount of overlay matrix to cold maintenance medium on ice. Mix well by pipetting several times.
21. Pipet overlay solution to plated hepatocytes, following the volume/well guidelines from Table 6.
22. Incubate for at least 2 hrs. before use. Replace maintenance medium daily.

Procedure for Cell Counting

To determine cell viability and viable cell yield with the Trypan Blue Exclusion Method, follow the directions below.

1. Add 50µL of 0.4% Trypan Blue Solution to 350µL of media. Or use pre-aliquoted 1:8 Trypan Blue:stock solution.
2. Add 100µL of cell stock. This makes a final 1:5 cell:stock dilution. This will be referred to as the 'dilution factor' in the formula below.
3. Determine cell viability using the formula below.

Live cell count	/	Total cell count	=	Viability
	/		=	%

4. Determine total viable cell yield using the formula below.

Viable cell count / Quadrants counted	x	Dilution factor	x	Hemocytometer factor	x	Current volume (mL)	=	Viable cell yield
/	x	5	x	10,000	x		mL =	

5. Use the formulas below to determine the volume of medium to add to your current cell stock to achieve the desired cell density (can be found on Table 7). The desired cell density varies for suspension assays but is most commonly 1.0×10^6 cells/mL. Use plating medium if plating your hepatocytes. Use maintenance medium for suspension assays.

Viable cell yield	/	Desired cell density*	=	Total volume needed
x 10^6 cells	/	x 10^6 cells/mL	=	mL

Total volume needed	-	Current volume	=	Volume to add to cell stock
mL -		mL=		mL

Table 7. Desired Cell Density by Species and Plate Format

Species	6-well	12-well	24-well	48-well	96-well
	Cell Density (10^6cells/mL)				
Human, Rat, Dog	0.9 – 1.1	0.8 – 1.0	0.7 – 0.9	0.6 – 0.8	0.9 – 1.1
Monkey	1.1 – 1.3	1.0 – 1.2	0.9 – 1.1	0.8 – 1.0	1.1 – 1.3
Mouse	0.5 – 0.7	0.4 – 0.6	0.3 – 0.5	0.2 – 0.4	0.5 – 0.7